

Vitreous Cytokine Profile Differences Between Eyes With Epiretinal Membranes or Macular Holes

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PURPOSE. Cytokines play an important role in cell signaling in inflammatory and repair processes, also within the posterior segment of the eye. These molecules are thus implicated in the pathophysiology of several vitreoretinal diseases. In the present study, we compared vitreous cytokine profiles in patients with idiopathic epiretinal membranes (ERMs) and idiopathic full-thickness macular holes (MHs) without epiretinal membranes.

METHODS. Native vitreous humor was collected during elective pars plana vitrectomy for the treatment of macular pathologies (group 1: ERM; group 2: MH) from patients without any other ocular or systemic disease. The concentrations of 43 chemokines and cytokines were measured in parallel by multiplex beads analysis. Intergroup comparisons were conducted using the Mann-Whitney *U* test and Bonferroni's correction, at a level of significance of $P < 0.0012$.

RESULTS. Vitreous samples from 31 patients with ERMs (group 1) and from 30 with MHs (group 2) were analyzed. For 12 of the tested cytokines (GM-CSF, MCP-1, MIF, CCL15, CCL20, CCL17, CX3CL1, CXCL10, CXCL16, and TGF- β -1, -2, and -3), no intergroup differences were revealed; for the other 31, the concentrations were higher in the ERM than in the MH group ($P < 0.0012$ in each case).

CONCLUSIONS. The vitreous levels of 72% of the tested cytokines were higher in ERM than in MH. This indicates that even in the absence of clinical markers, activation of inflammatory and profibrotic mechanisms is implicated in the progression of ERMs. Although frequently used as such in the past, eyes with ERMs should be considered with caution as a healthy control group.

Keywords: biomarkers, chemokines, cytokines, cytokine profiles, epiretinal membrane, fibrosis, gliosis, interleukins (IL), macular hole, tumour necrosis factor (TNF)

Cytokines are cell-signaling proteins that mediate diverse physiological processes. They also play a crucial role in various inflammatory processes, as well as in wound healing and fibrotic scarification.¹ Fibrosis is a process that is characterized by an exuberant formation of connective tissue, which entails the production of collagens and glycosaminoglycans by activated fibroblasts. In the retina, tissue-resident cells, such as glia and macrophages, initiate fibrosis by the release of soluble factors,^{2,3} among which TGF- β is one of the best characterized.^{1,4} Other profibrotic mediators include the platelet-derived growth factor (PDGF), IL-4, IL-6, IL-13.^{1,2,5} All of these cytokines stimulate the production and deposition of collagen by fibroblasts and promote their activation and differentiation into myofibroblasts.^{1,2,5} Müller glial cells are known to activate fibroblasts and to promote fibrotic changes within the eye, such as proliferative vitreoretinopathy (PVR)^{3,6} and the formation of epiretinal membranes (ERMs).^{7,8} Epiretinal membranes are characterized by the growth of fibrocellular tissue along the inner limiting membrane (ILM), which can lead to metamorphopsia and visual loss. The most frequently encountered, so-called idiopathic ERMs, are not linked to any

other ocular disease process and their pathogenesis remains unidentified. Macular holes (MHs), on the other hand, are full-thickness foveal defects, extending from the ILM to the RPE. The release of vitreomacular traction (VMT) as the underlying pathomechanism of idiopathic macular holes^{9,10} leads to degenerative and atrophic changes, including the formation of prominent, cystoid spaces at the edge of the MH, which may result in its enlargement without detectable scar formation in more than 50% of the cases.^{11,12} In the remaining instances, a horizontal traction, which is represented by a hyperreflective band in optical coherence tomography on the inner retinal surface with or without wrinkling of the retina, is visible. This phenomenon is probably the consequence of either ILM-contraction or of the formation of a thin ERM. In these cases, enlargement of the MH, resulting from both a contraction of the ERM and atrophic changes, is to be expected.¹³

In various ocular diseases, such as proliferative diabetic retinopathy, retinal vein occlusion, uveitis, retinal detachment, high myopia, and lymphoma, the vitreous levels of many cytokines are elevated.^{14–18} In several of the published reports, patients with vitreomacular pathologies, such as MHs or ERMs,



were used as controls,^{14,16–18} even though the vitreous cytokine profiles in these vitreoretinal interface diseases have not as yet been well characterized or compared.

The aim of the present study was therefore to compare the vitreous cytokine profiles in patients with either idiopathic ERMs or idiopathic full-thickness MHs, with a view to assessing the degree to which fibrotic and inflammatory processes contribute to the progression of these diseases.

PATIENTS AND METHODS

Patients

This prospective clinical study included patients with symptomatic idiopathic ERMs or MHs requiring vitrectomy. Surgery was performed at the Berner Augenklinik am Lindenhofspital in Bern with the informed, written consent of the individuals concerned. Clinical data pertaining to the systemic and ophthalmologic diagnosis, the treatment strategy, and the duration of the ocular symptoms were documented. Patients with systemic comorbidities, that is, with diabetes mellitus, a history of intraocular surgery (other than uneventful phacoemulsification for senile cataract, performed minimally 6 months before vitrectomy), ocular trauma, vitreous hemorrhaging, uveitis, glaucoma, or any concomitant retinal pathology, were excluded. Vitreous samples from patients with coexisting ERMs and MHs in the same eye were also collected and the data is presented in the Supplementary Material. The study was approved by the local Institutional Ethics Board of the University of Bern, Switzerland (registration number: 152/08) and fully complied with the tenets of the Declaration of Helsinki.

Collection of Vitreous Samples

Aliquots of native vitreous (approximately 0.5 mL) were collected between January 2012 and April 2014 at the onset of 23-G pars plana vitrectomy, after the insertion of the ports and before opening the infusion line. The samples were stored at -20°C for up to 2 months and thereafter at -80°C until all of the specimens had been collected for the in-parallel analysis.

Cytokine Analysis

The vitreous samples were analyzed using a multiplex system (Bio-Plex 100 array reader with Bio-Plex Manager software [version 6.1]; Bio-Rad, Hercules, CA, USA). Using this system, multiple analytes can be detected and quantified in parallel in a single small sample volume. In the present study, the concentrations of 43 cytokines in each vitreous sample (Table) were quantified. All analytic procedures were performed according to the manufacturer's guidelines. In short, magnetic microspheres that were tagged with a fluorescent label were coupled to specific capture antibodies and mixed with samples containing unknown quantities of the cytokines. Biotinylated detection antibodies and Streptavidin R-Phycoerythrin were then introduced. The mixture was analyzed by flow cytometry. The instrument's two lasers identify the microsphere type and quantify the amount of bound antigen. A concentration standard was run in parallel on each test plate. The measurements were performed in a blinded manner by a laboratory technician who was experienced in the execution of the technique.

Because the number of the selected cytokines was large, the data were presented in the form of a color-coded heat map in which the lowest values were depicted in light green and the highest in red.

Statistical Analysis

Numerical data are presented as mean values together with the SD. The concentrations of several of the cytokines lay below the curve fit of the standards. To avoid the bias that would have been introduced by excluding these values, they were set at half of the lowest quantified level for the cytokine in question. Outliers (values that lay 1.5 box lengths beyond the box edges) were, on the basis of box-plot inspection, preexcluded from the statistical analysis.

The Shapiro-Wilk test was instrumented to establish whether the data were normally distributed. Because the criteria for a normal distribution were not satisfied, the nonparametric Mann-Whitney *U* test was implemented for the intergroup comparisons. To avoid the introduction of type I errors, Bonferroni's correction was applied to the preset level of significance ($P < 0.05$).¹⁹ For this purpose, the *P* value was divided by the number of the cytokines whose concentrations were determined ($n = 43$), which resulted in a shift of the level of significance from $P < 0.05$ to $P < 0.0012$. The statistical analysis was performed using SPSS (version 23.0; IBM SPSS Statistics, Armonk, NY, USA).

RESULTS

Patients

The two groups of patients (ERM: $n = 31$; MH: $n = 30$) were composed of individuals of comparable age at the time of inclusion (73 ± 8.5 vs. 71 ± 8.2 years in the ERM and MH groups, respectively [$P = 0.3$]). According to the medical histories of the patients, the interval that elapsed between the first reported symptoms and surgery was longer than 6 months in 94% of the individuals in the ERM group and 27% of those in the MH group. Symptoms of less than 3 months were reported by 73% of patients with MH. Cataract surgery had been performed minimally 6 months before inclusion in 26% of the individuals in the ERM group and in 13% of those in the MH group.

Cytokine Analyses

The absolute concentrations of the cytokines in the vitreous fluid spanned a broad range that was specific for each type (Table).

The levels of 31 of the cytokines (72%) were higher in the ERM than in the MH group (Table; Figs. 1, 2). (These cytokines were the following: CCL21, CXCL13, CCL27, CXCL5, CCL24, CCL26, CCL11, CXCL6, CXCL1, CXCL2, CCL1, IFN- γ , IL-10, IL-16, IL-1 β , IL-2, IL-4, IL-6, IL-8, CXCL11, CCL8, CCL7, CCL13, CCL22, CXCL9, CCL3, CCL19, CCL23, CXCL12, CCL25, and TNF- α .) The differences between the two groups remained significant for each of the 31 cytokines also after the exclusion of outliers. For the other 12 cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF], CXCL10, MCP-1, MIF, CCL15, CCL20, CCL17, CX3CL1, CXCL16, and TGF- β -1, -2, and -3), the levels in the two groups were similar.

Interestingly, the distribution of the 43 cytokines in Figure 1 suggests a specific cytokine profile in the vitreous, with higher levels in the ERM group.

The largest intergroup differences, which were on the order of 10-fold, were registered for CCL27, CCL26, CXCL6, IFN- γ , IL-10, and CXCL11. These data are separately displayed in Figure 2. For each of these six cytokines, the concentrations lay in the lower range (below the cutoff level) in 20% of the ERM- and in 63% to 100% of the MH-group samples (Fig. 3).

In 31.9% of the measured samples in both groups, the concentrations of the cytokines lay below the limit of detection

TABLE. Overview of All 43 Cytokines Analyzed With a Multiplex System (Bio-Plex) From Vitreous Samples of Patients With ERMs (Group 1) and MHs (Group 2)

Cytokine	ERM						MH						Kruskal-Wallis H-Test,		Confidence Interval,	
	Mean	SD	Se	Range			n	Mean	SD	Se	Range		ERM - MH	n	ERM - MH	ERM - MH
				Min.	Max.	Min.					Max.					
CCL121	627.80	333.67	61.96	12.89	1427.38	29	330.70	150.18	27.89	74.26	707.10	29	0.0005		163.21 to 370.50	
CXCL113	0.77	0.46	0.09	0.05	1.66	29	0.14	0.31	0.06	0.02	1.26	30	0.0005		0.40 to 0.90	
CCL127	6.06	7.47	1.39	0.00	23.56	29	0.43	1.17	0.22	0.00	5.74	29	0.0005		0.65 to 5.46	
CXCL5	80.00	51.04	9.32	15.25	209.04	30	16.21	3.82	0.70	15.25	33.95	30	0.0005		39.04 to 57.63	
CCL124	15.19	7.25	1.32	5.73	33.57	30	5.81	5.33	0.99	0.31	21.45	29	0.0005		5.93 to 11.94	
CCL126	1.63	1.28	0.24	0.01	4.09	29	0.04	0.08	0.02	0.01	0.39	29	0.0005		1.51 to 2.08	
CCL11	5.37	2.00	0.37	1.14	9.93	30	0.63	0.71	0.13	0.21	2.15	29	0.0005		4.25 to 5.42	
CX3CL1	29.18	13.27	2.42	3.75	57.52	30	19.63	11.07	2.06	5.17	41.11	29	0.005		3.52 to 16.05	
CXCL6	2.67	2.00	0.36	0.09	6.87	31	0.09	0.00	0.00	0.09	0.09	30	0.0005		1.67 to 3.27	
GM-CSF	37.59	14.97	2.69	2.59	79.46	31	28.44	10.86	2.02	5.17	51.50	29	0.015		1.37 to 15.01	
CXCL1	38.73	11.17	2.01	16.75	60.59	31	5.22	9.25	1.69	1.43	43.08	30	0.0005		29.50 to 40.07	
CXCL2	7.21	7.17	1.29	0.83	32.67	31	1.35	2.29	0.42	0.83	13.39	30	0.0005		1.46 to 7.36	
CCL1	5.50	5.34	0.96	1.53	19.51	31	1.53	0.00	0.00	1.53	1.53	30	0.0005		0.00 to 3.02	
IFN-γ	4.01	4.61	0.83	0.36	17.21	31	0.36	0.00	0.00	0.36	0.36	30	0.0005		1.00 to 3.38	
IL-10	4.73	1.75	0.32	1.02	8.65	30	0.43	1.10	0.20	0.15	6.03	30	0.0005		3.82 to 5.16	
IL-16	32.81	20.62	3.83	8.06	87.17	29	5.80	18.62	3.46	0.75	100.37	29	0.0005		18.69 to 38.51	
IL-1β	0.89	0.41	0.08	0.13	1.75	28	0.10	0.24	0.04	0.02	1.14	30	0.0005		0.67 to 0.96	
IL-2	0.68	0.47	0.09	0.11	1.55	30	0.07	0.09	0.02	0.05	0.54	30	0.0005		0.31 to 0.78	
IL-4	3.21	2.46	0.45	1.30	7.84	30	1.36	0.37	0.07	1.30	3.30	30	0.0005		0.00 to 1.30	
IL-6	5.33	3.30	0.63	0.50	11.63	27	0.80	1.55	0.29	0.01	6.13	28	0.0005		2.64 to 5.49	
IL-8	15.98	18.04	3.24	0.51	74.92	31	0.92	1.07	0.21	0.03	3.79	26	0.0005		4.14 to 16.55	
CXCL10	45.59	25.79	4.87	4.32	115.32	28	25.53	21.78	4.12	1.92	71.02	28	0.002		7.51 to 32.16	
CXCL11	1.13	0.57	0.10	0.00	2.09	29	0.03	0.15	0.03	0.00	0.81	30	0.0005		0.80 to 1.19	
MCP-1	435.11	162.94	31.95	24.49	675.89	26	391.28	151.42	28.12	191.17	748.86	29	0.20		-38.18 to 137.10	
CCL8	2.43	1.45	0.27	0.22	5.24	29	0.63	0.55	0.10	0.06	1.75	29	0.0005		0.97 to 2.46	
CCL7	7.79	8.24	1.48	1.53	29.09	31	1.53	0.00	0.00	1.53	1.53	30	0.0005		0.00 to 5.67	
CCL13	1.03	0.44	0.08	0.29	1.97	31	0.14	0.00	0.00	0.14	0.14	30	0.0005		0.72 to 0.96	
CCL22	10.13	5.39	1.02	3.28	21.54	28	2.89	3.01	0.55	0.83	12.69	30	0.0005		4.87 to 7.64	
MIF	12444.39	7550.51	1453.10	1842.88	30008.1	27	12902.38	9106.93	1721.05	1898.82	39365.81	28	0.83		-4462.95 to 4408.54	
CXCL9	11.28	8.18	1.52	1.68	29.88	29	1.55	1.85	0.36	0.05	6.27	27	0.0005		5.05 to 10.68	
CCL3	1.18	0.40	0.08	0.62	2.12	27	0.13	0.11	0.02	0.01	0.37	28	0.0005		0.85 to 1.17	
CCL15	668.80	517.26	97.75	16.16	1877.75	28	284.86	187.39	36.06	76.03	770.48	27	0.001		95.98 to 494.18	
CCL20	4.11	1.75	0.33	0.92	8.08	28	3.57	2.58	0.48	0.36	9.26	29	0.21		-0.41 to 1.91	
CCL19	10.02	4.54	0.84	3.18	18.90	29	1.88	1.81	0.33	1.56	11.45	30	0.0005		5.11 to 10.12	
CCL23	13.77	7.53	1.42	0.18	36.97	28	4.03	7.05	1.29	0.18	25.13	30	0.0005		7.02 to 13.40	
CXCL16	824.59	270.39	48.56	11.52	1174.60	31	653.61	231.50	42.27	222.48	1163.14	30	0.004		69.87 to 335.60	
CXCL12	124.77	58.33	10.83	17.29	236.30	29	42.68	22.56	4.19	13.54	108.36	29	0.0005		54.90 to 108.28	
CCL17	0.63	0.11	0.02	0.61	1.22	31	0.61	0.00	0.00	0.61	0.61	30	0.33		0 to 0	
CCL25	63.97	33.38	6.20	25.38	143.59	29	23.58	11.32	2.18	4.92	50.87	27	0.0005		22.83 to 43.74	
TNF-α	7.09	3.55	0.66	1.22	16.34	29	2.59	1.40	0.26	0.68	6.45	29	0.0005		2.89 to 5.62	
TGF-β1	13.67	21.42	3.91	0.95	65.82	30	7.71	19.78	3.67	0.95	94.24	29	0.33		0 to 0	
TGF-β2	951.06	593.25	110.16	152.82	2558.13	29	942.57	402.91	74.82	344.36	1754.20	29	0.68		-289.31 to 209.25	
TGF-β3	1.04	1.70	0.30	0.10	7.22	31	1.48	3.54	0.65	0.10	18.36	30	0.95		0 to 0	

Max., maximum; Min., minimum.



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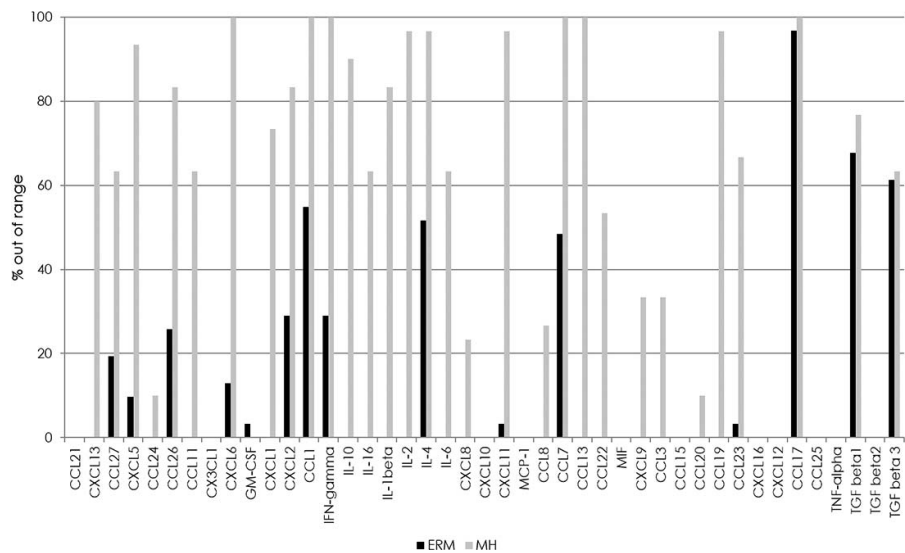


FIGURE 3. Percentage of samples with nondetectable cytokine levels (out of range) in the ERM and MH groups.

approach the lower limits of the test system's sensitivity. Hence, it may be necessary to develop new modes of data analysis before we can move forward in this area. It would be conceivable, for example, to generate a microarray-based system to estimate the whole intercellular communication environment, a cytokinome comparable with the proteome

generated by proteomic microarrays. We chose also an alternative approach to present and compare the assembled data for the selected cytokines en bloc, in the form of a color-coded heat map (Fig. 4), which facilitates interpretation of intergroup outcomes using visualization technique instead of a bulk of quantitative data.

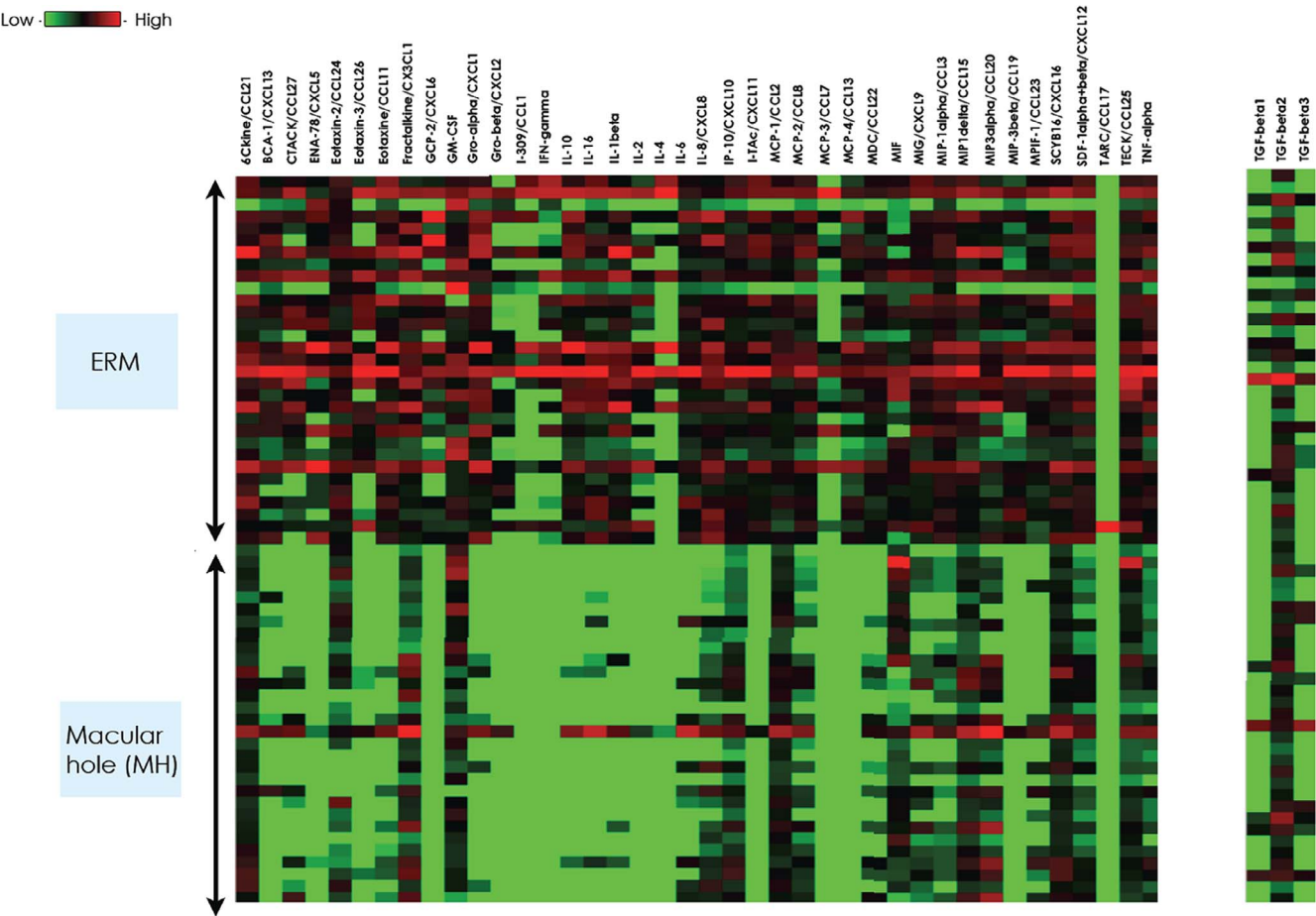


FIGURE 4. Visualization of the intergroup differences using a heat map chart for all 43 cytokines (light green indicates low levels, whereas dark red indicates elevated levels).

In addition, we have confirmation of our data in that at least some of our measured vitreal cytokine levels show levels that are comparable to already published data using the Bio-Plex technique (i.e., for IL-1 β , IL-4, IL-8, and TNF- α).^{15,21,22}

Vitreomacular interface disorders, such as vitreomacular traction, can result in the formation of MHs. The persistence of such a disturbance can lead to the growth of ERMs, which may or may not be associated with the formation of MHs.²³ It is not currently known whether ERMs and MHs result primarily from a vitreal or from a retinal pathology. Our data suggest that ERMs and MHs progress along different pathophysiologic courses. Acute mechanical traction is believed to be the leading cause of MHs, which is released on their clinical manifestation. By this time, and thus at the time of surgery, the cytokine response to the mechanical traction has abated. In the case of ERMs, the mechanical traction is chronic, and the cytokine response thereto is sustained until, and is still evident at, the time of surgery, and may contribute to the progression of the disease. Because the vitreal samples were collected during the chronic phase of the diseases, we can do no more than speculate on the cell-signaling roles of the cytokines in the pathologic processes. Because the cytokine levels for MHs that coexisted with ERMs in the same eye were comparable to those that were characteristic of ERMs (data presented in the Supplementary Material), it is conceivable that the vitreomacular-traction-induced formation of MHs and ERMs is associated with a trauma-induced activation of cytokine production, which rapidly abates in the former pathology (MHs) but persists in response to a persistent traction in the latter (ERMs).

From a histopathologic point of view, the fibrotic process that is associated with the growth of ERMs involves diverse cell types, including hyalocytes, fibroblasts, and glia cells,²⁴ which are activated by different fibroblast-signaling pathways and which participate in the secretion of extracellular-matrix components.²⁵ Activated Müller glia cells contribute to the formation of ERMs. Transforming growth factor- β induces the differentiation of these cells into a fibroblast-like phenotype and promotes their migration.^{7,8} Characteristic cytokine profiles for the differentiation of fibroblasts and retinal glia are unknown, but we assume activated fibroblasts as a source, but not so evidently Müller glia cells, in the chronic phase of ERM formation. The cytokine profiles in our study are commensurate with an activation of fibrotic and inflammatory processes, which are associated with elevated levels of IL-6, IL-4, IL-1 β , TNF- α , IL-10, IL-2, and INF- γ (among others).^{2,26–28} In our study, five of the six chemokines and cytokines with the largest intergroup differences (CCL27, CCL26, CXCL6, INF- γ , and CXCL11) are proinflammatory and play a major role in immunomodulation and inflammation, indicating a central role of inflammatory mechanisms in epiretinal membrane formation. Additionally, IL-10 was initially understood as a TH2 cytokine with regulatory properties in inflammatory processes, but meanwhile it was revealed that lymphocytes, macrophages, dendritic, natural killer, and mast cells also express IL-10.²⁹ Its contribution to fibrosis has remained controversial.²⁹ The upregulation of vitreal IL-10 levels in our ERM group might indicate its immunomodulatory role in response to the proinflammatory cytokines and chemokines so as to reconstitute a physiological balance. In the vitreal humor of eyes with MHs, the concentrations of proinflammatory cytokines did not attain relevant levels.

Interestingly, TGF- β -2 was the most abundant TGF subtype in our study, whereas in previous reports, TGF- β -1 was identified as the more relevant cytokine in the formation of ERMs.^{24,30} Transforming growth factor- β -1 and the nerve growth factor were shown to play a crucial role in fibroblast activities and were detected in the vitreal humor of each of the eight patients with idiopathic ERMs.³⁰ Kohno et al.²⁵ demon-

strated that TGF- β -2 stimulates the differentiation of hyalocytes into myofibroblasts. Interestingly, Zhao et al.³¹ reported hyalocytes and retinal Müller cells to be the predominant cell types in idiopathic ERMs. Transforming growth factor- β -2 thus appears to be an important growth factor in the evolution of ERMs and in their contraction.^{25,32} In our study, the vitreal levels of TGF- β -2 in the ERM group were higher than those of TGF- β -1 and -3. However, such was the case also in the MH group, which is a seemingly unaccountable finding. Nevertheless, the question remains unanswered whether cytokine levels in general, namely TGF- β levels are similar throughout the whole vitreous in these macular pathologies.

In conclusion, an activated cytokine profile exists in the vitreal humor of patients with ERM but not in that of individuals with MHs during the chronic phase of the diseases. The lack of such a response in the MH group reflects the absence of a fibrotic switch after the induction of the initial mechanical trauma, the nature of which may have been the same in both pathologies.

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